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The endothelin 1_A receptor antagonist BSF 302146 is a potent inhibitor of neointimal and medial thickening in porcine saphenous vein–carotid artery interposition grafts

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Objective: Late saphenous vein graft failure after coronary artery bypass graft surgery is initiated by medial thickening and neointima formation, both of which are mediated by the proliferation of vascular smooth muscle cells. Because porcine vein grafts contain high levels of endothelin 1 receptor subtypes and endothelin 1 promotes the proliferation of vascular smooth muscle cells, the effect of administration of the endothelin 1_A receptor antagonist BSF 302146 ([+]-[S]-2-[4,6-dimethyl-pyrimidin-2-yloxy]-3,3-diphenyl-butanoic acid) on porcine vein graft thickening was investigated.

Methods: Saphenous vein–carotid artery interposition grafting was performed in 4 groups of large white pigs (30-35 kg, n = 10 for each group). BSF 302146 was administered orally (3, 10, and 30 mg $\cdot$ $kg^{-1} \cdot d^{-1}$) for 4 weeks to one group of pigs, and placebo was administered to the other group (control animals). Pigs were then anesthetized, and the grafts were removed and fixed at 100 mm Hg with 4% paraformaldehyde. Histologic sections were prepared, and graft morphometry was carried out by using computer-aided planimetry.

Results: In vein grafts from animals treated with BSF 302146 compared with grafts from control animals (untreated), there were significant dose-dependent reductions in the increase in medial thickness and neointimal thickness, an increase in luminal area, and a decrease in proliferating cell nuclear antigen–positive cells in the medial-intimal area.

Conclusions: The administration of BSF 302146 reduces graft thickening and promotes positive remodeling through an endothelin 1_A–mediated effect on vascular smooth muscle cell replication. The administration of this endothelin 1_A receptor antagonist might therefore be therapeutically effective in preventing late vein graft failure in patients undergoing coronary artery bypass grafting.

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Vein graft thickening and superimposed atherogenesis is the main cause of late failure after coronary artery bypass graft (CABG) surgery with autologous saphenous vein.[1-3] Vein graft thickening is determined on the basis of increased medial thickening and neointima formation, both of which involve the proliferation and migration of vascular smooth muscle cells (VSMCs).[4] Superimposed on these rapid events is atherogenesis, which ultimately leads to graft occlusion in as many as 50% of patients within 10 years.[1-3] Apart from aggressive lipid-lowering therapy,[5] there is no effective therapeutic intervention for late vein

graft failure,[4] and as such, this constitutes a considerable clinical problem that needs to be urgently resolved.

Of the many factors implicated in vein graft disease, endothelin-1 (ET-1) promotes VSMC migration and proliferation[6] and restenosis after balloon injury.[7-10] Furthermore, there are high endothelin 1_A (ET_A) receptor densities in the tunica media and neointima of porcine saphenous vein grafts,[11] suggesting a role for ET-1 in mediating vein graft thickening. ET_A receptor density was also found to be greater in ungrafted saphenous vein compared with in the carotid artery,[11] suggesting that the saphenous vein might be more susceptible to ET-1–driven VSMC replication and migration than arterial conduits. ET-1 also plays a contributory role in atherogenesis,[12] the ultimate cause of vein graft failure.[2,3]

These observations pointed to the potential use of an ET_A receptor antagonist to reduce graft thickening after CABG. The effect of oral administration of the ET_A receptor antagonist BSF 30214613[13] on vein graft thickening was studied in a porcine model of saphenous vein–carotid artery interposition grafting to test this hypothesis.[14,15]

Materials and Methods

Surgical Procedure

Studies were performed with Landrace pigs weighing 30 to 35 kg, which received humane care according to the Home Office Animal Care regulations. All animals underwent saphenous vein into carotid artery interposition grafting.[14] Anesthesia was induced with ketamine (30 mg) and atropine (0.6 mg) administered intramuscularly. After endotracheal intubation, anesthesia was maintained with halothane and oxygen, and animals were allowed to ventilate spontaneously throughout. Heparin sodium (1 mg/kg) was administered intravenously, and a single dose of 250 mg of benzyl penicillin was administered intramuscularly before skin incision. A longitudinal incision was made on the outer aspect of the hind limb. Approximately 10 cm of the vein was then dissected free of surrounding tissue by using a no-touch technique, and all side branches were secured with a 6-0 Prolene ligature (Ethicon Inc). The vein was removed from the animal, rinsed in iso-osmotic sodium chloride solution (0.9 g/L) containing 2 IU/mL heparin and 50 μg/mL glyceryl trinitrate, and stored in the same solution at room temperature (23°C) until needed.

A longitudinal neck incision was made just medial to the sternomastoid muscle, and the common carotid artery was carefully dissected from the internal jugular vein and vagus nerve within the carotid sheath. A 3-cm segment of the common carotid artery was isolated between vascular clamps and excised, beveling the cut ends obliquely to 45°. The saphenous vein was cut to the appropriate length, reversed, and similarly beveled, and an end-to-end anastomosis of the vein to the common carotid artery was carried out by using a continuous 7-0 Prolene suture. Animals were extubated and, when in a satisfactory condition, returned to their pens and fed a normal chow diet.

The specific ET_A receptor antagonist BSF 302146 ([+]-[S]-2-[4,6-dimethyl-pyrimidin-2-yloxy]-3,3-diphenyl-butanoic acid, Knoll AG) was then administered to the pigs (3, 10, and 30 mg/kg; n = 10 for each drug dose) daily for 28 days mixed with a portion of mashed potato to ensure complete intake of the drug. The control group (n = 10) was fed placebo. After 1 month, pigs were reanesthetized as above, grafts were removed (including 1-cm segments of the proximal and distal carotid arteries), and the pressure was fixed ex vivo at 100 mm Hg by using 6% paraformaldehyde buffered with phosphate-buffered saline and postfixed in the same solution for 24 hours before being rinsed in phosphate-buffered saline and processed for wax embedding, histology, and planimetry.[14,15]

Histology and Immunocytochemistry

Histology and immunocytochemistry were carried out as previously described.[14,15] For histology, sections were dewaxed, rehydrated, and stained with hematoxylin and eosin or Miller's elastic van Gieson stain. For immunocytochemistry, sections were dewaxed, rehydrated, and treated with hydrogen peroxide in methanol to remove endogenous peroxidase, and the following staining was carried out. For VSMCs, sections were treated with horse serum diluted 1:3 with Tris-buffered saline (TBS; pH 7.4), drained, and incubated with smooth muscle actin clone 1A4 monoclonal (Dako) at 1:1000 overnight at 4°C. After washing in TBS, the sections were treated with biotinylated goat anti-mouse (Dako) at 1:400 and, after washing in TBS, treated with streptavidin biotin complex/horseradish peroxidase (Dako) followed by 3,3'-diaminobenzidine. The nuclei were counterstained with diluted Harris hematoxylin, dehydrated, and mounted.

For proliferating cell nuclear antigen (PCNA), sections were microwaved in citrate buffer, quenched in 1:3 horse serum in TBS, and then incubated with PCNA antibody diluted 1:100 overnight at 4°C. Sections were washed and then treated with 1:400 biotinylated goat anti-mouse antibody, followed by streptavidin biotin complex/horseradish peroxidase. Visualization was achieved with 3,3'-diaminobenzidine, and then after counterstaining with diluted hematoxylin and eosin, sections were dehydrated and mounted. PCNA was apparent as dark brown staining of nuclei, whereas cytoplasmic nuclei were stained blue.

Morphometric Analysis

Vessel wall dimensions were measured by means of computer-aided planimetry with an Olympus BH-2 microscope with a color video camera head (JVC TK-870E) coupled to a Microscale TM/TC image analysis system (Digithurst Ltd). The area enclosed by the endothelium and the internal elastic lamina defined the intima, and the area between the internal and external elastic lamina defined the media. Luminal, intimal, and medial perimeters and areas were computed by using the luminal boundary and the internal and external elastic lamina as delimiters, and mean values were then calculated for all sections from the same graft. Average intimal, medial, and total vessel wall thickness was derived from the area and perimeter data for 5 sections from each graft, assuming that the sections consisted of circular profiles, which was a valid assumption because the tissues were fixed at normal perfusion pressures. The number of cells positive for PCNA, as well as PCNA-negative cells in the intima and media together, were counted in 4 luminal fields (at 40× magnification) for each section (5 sections per graft), and the percentage was calculated.[14,15]

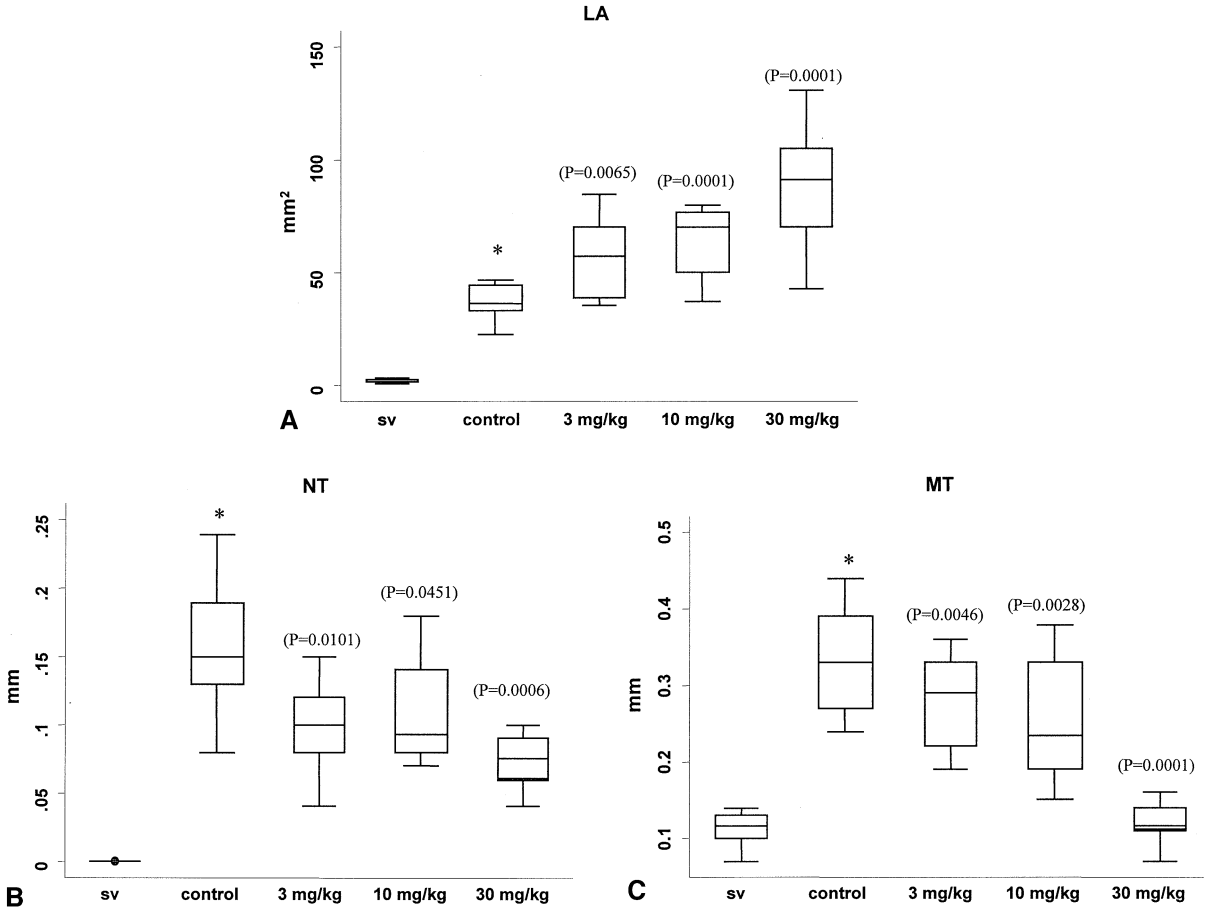

Figure 1. Planimeteric analysis of vein grafts from pigs to which BSF 302146 was administered orally once daily for 1 month before explantation: A, luminal area *(LA)*; B, neointimal thickness *(NT)*; and C, medial thickness *(MT)*. Data are expressed as medians and interquartile ranges with *P* values (n = 10). *Asterisks* denote statistical significance when comparing ungrafted saphenous vein with vein grafts from placebo-treated pigs (Mann-Whitney test with a Bonferroni adjustment). By using the Kendall τ-b test, significant dose-response relationships were obtained for BSF 302146 versus luminal area ($P < .001$), neointimal thickness ($P < .001$), and medial thickness ($P < .001$).

Data Analysis and Statistics

Data were collated and analyzed with Microsoft Excel, and nonparametric statistical analysis was carried out by using an Intercooled Stata 8 statistics package (Stata Corp). Although data did not appear to be skewed, the Bartlett test for equality of variance (a necessary assumption for a 1-way analysis of variance) was significant, indicating that nonparametric methods of analysis were required. Thus values are expressed as medians and interquartile ranges and graphically as whisker box plots. When the effects of drug treatments on vein graft planimetry were compared with those in placebo-fed control animals, the Kruskal-Wallis test, a nonparametric version of analysis of variance, was applied. Mann-Whitney tests were then applied to test the statistical significance of each of the treated groups individually for each drug dose compared with placebo-treated control animals. A Bonferroni adjustment was then used (0.05/3). A Kendall τ-b test was used as a measure of correlation for ordinal categoric data, which takes into account the testing of whether drug effects were dose dependent.

Results

All grafts were patent 4 weeks after implantation. There were statistically significant increases in neointimal thickness, medial thickness, and luminal areas in saphenous vein grafts 4 weeks after implantation compared with ungrafted saphenous veins (Figures 1 and 2). There was a dose-dependent and statistically significant increase in luminal area in response to BSF 302146 administration compared with that seen in placebo-fed control animals (Figures 1 and 2). There was a dose-dependent and statistically significant decrease in medial thickness in vein grafts from pigs treated with BSF 302146 compared with that in vein grafts from

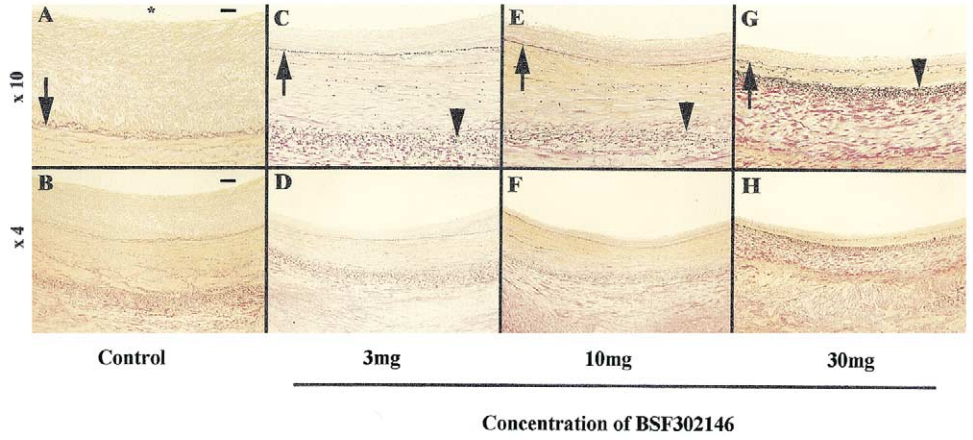

Figure 2. Effect of oral administration of the ET_A antagonist (BSF 302146) on neointima formation. Elastin van Gieson–stained serial transverse sections of a representative control vein graft (A and B) and grafts 4 weeks after administration of 3 mg (C and D), 10 mg (E and F), and 30 mg (G and H) of ET_A antagonist. *Arrows* and *arrowheads* in panels A, C, E, and G indicate the internal and external elastic lamina, respectively. The scale bar in panel A applies to panels C, E, and G and represents 100 μm. The scale bar in panel B applies to panels D, F, and H and represents 250 μm. The *asterisk* indicates the lumen.

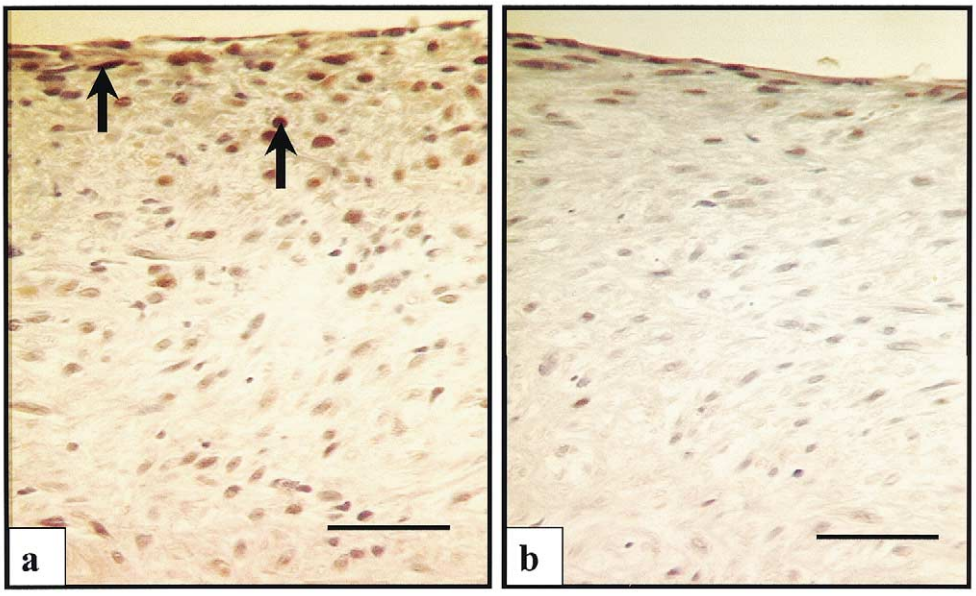

Figure 3. Representative photomicrographs of PCNA-positive staining (brown, examples *arrowed*) and PCNA-negative staining (blue) in the intimal-medial regions of vein grafts 1 month after implantation from placebo-treated pigs (A) and pigs to which 30 mg/kg BSF 302146 was administered (B). Scale bar = 50 μm.

placebo-treated animals (Figures 1 and 2). Neointimal thickness in vein grafts from pigs treated with BSF 302146 was reduced compared with that in vein grafts from untreated animals, which again was statistically significant at 3 and 30 mg/kg but not at 10 mg/kg. However, without applying a Bonferroni adjustment, the effect at 10 mg/kg was statistically significant, and there was also a highly significant dose-response relationship between BSF 302146 and the reduction in neointimal thickness (Figure 1). A statistically significant lower percentage of PCNA-positive (proliferating) cells was detected in the intimal-medial regions of the grafts obtained from animals treated with BSF 302146 compared with placebo-fed control animals (Table 1 and Figure 3). At 3 and 10 mg/kg BSF 302146, there were

TABLE 1. Percentage of cells with PCNA-positive nuclei relative to PCNA-negative nuclei in the intimal-medial regions of vein grafts from pigs treated with the ET_A antagonist BSF 302146 compared with percentages in control animals

Placebo	3 mg/kg	10 mg/kg	30 mg/kg
19.1 (15.9-23.0)	12.45 (11.8-13.6),* $P = .0001$	10.25 (8.0-13.15),* $P = .0001$	8.7 (7.4-8.2),* $P = .0001$

Data are expressed as medians (interquartile ranges; n = 10).
*Statistical when comparing vein grafts from animals treated with antagonist with those from control animals (untreated; Mann-Whitney test with a Bonferroni adjustment)

TABLE 2. Cell density (number of cells per square millimeter) in the intimal-medial region of vein grafts from pigs treated with BSF 302146 compared with density in control animals

Placebo	3 mg/kg	10 mg/kg	30 mg/kg
7500 (7200-7580)	7300 (7050-7442), NS	7568 (7320-7820), NS	7090 (6900-7240),* $P = .0028$

Data are expressed as medians (interquartile ranges; n = 10). *NS,* Not significant.
*Statistical when comparing vein grafts from animals treated with antagonist with those from control animals (untreated; Mann-Whitney test with a Bonferroni adjustment)

no statistically significant differences in cell density compared with that seen in untreated control animals, but at 30 mg/kg, there was a slight but still statistically significant reduction in cell density (Table 2).

Discussion

The present study demonstrates that the oral administration of an ET_A receptor antagonist, BSF 302146, promotes a marked and dose-dependent inhibition of medial and intimal thickening, increased luminal area, and decreased PCNA-positive cells in porcine saphenous vein grafts. In the same pig model, we previously found a pronounced expression of ET_A receptors in the neointima and the tunica media of saphenous vein grafts 1 month after implantation.[11] Because ET-1 promotes the proliferation and migration of VSMCs, two central processes in medial thickening and neointima formation,[6] it is concluded that the effect of the antagonist is mediated through the inhibition of these processes. Other studies have also demonstrated the inhibitory capacity of ET-1 receptor antagonists on neointima formation after balloon injury in other animal models and in an in vitro organ culture model of the human saphenous vein.[6-10,16] Mediation of the effects of BSF 302146 by ET_B receptors is unlikely because BSF 302146 has a 78-fold greater affinity for ET_A compared with ET_B receptors,[13] and ET_B receptors are associated principally with microvessels and nerves rather then the neointima or medial regions of porcine vein grafts.[11]

It has been suggested that the saphenous vein graft is particularly vulnerable to pathologic attack by ET-1.[6,17] First, the pig saphenous vein contains a greater density of ET_A receptors than the carotid artery.[11] This would render the saphenous vein intrinsically susceptible to ET-1–mediated graft thickening in the present model. Certainly the incidence of graft failure is far less when arteries (eg, internal thoracic artery) are used in CABG compared with saphenous vein.[2,3] Furthermore, because immediately after implantation saphenous vein grafts are subjected to pulsatile pressure and high shear stress,[17] which in turn elicit ET-1 expression in vascular tissues and cells,[18-20] it is likely that hemodynamic forces will contribute to high intragraft levels of ET-1. Indeed, it has been demonstrated that there is a marked endogenous upregulation of ET-1 expression in porcine vein grafts 1 month after implantation.[11] Platelet and leukocyte adhesion is also an immediate consequence of vein graft implantation.[21] In turn, these cells release ET-1 itself,[21,22] as well as releasing a number of factors, including cytokines, that promote the release of ET-1 from vascular cells.[23] Thus coupled with the high levels of ET_A receptors, the locally high concentrations of ET-1 associated with vein graft implantation not only render the graft susceptible to ET-1–induced pathology but also explain the present emphatic effect of the ET-1 antagonist.

In the present study there was marked positive remodeling in response to the administration of BSF 302146 in that luminal area increased in a dose-dependent manner. One possible explanation for this is that the ET-1 antagonist might attenuate matrix protein formation and deposition, in particular collagen and elastin. It has been demonstrated that ET-1 is a potent stimulator of collagen synthesis (type I and III) in vascular tissues and cells.[12] The prevention of matrix deposition might therefore render the graft more susceptible to distension and therefore to increased luminal area. Although there was no change in the density of VSMCs (an index of matrix protein deposition[14,15]) after administration of BSF 302146 at 3 and 10 mg/kg, but a decrease with 30 mg/kg, an earlier effect of the antagonist on extracellular

matrix protein deposition cannot be discounted and warrants further study. ET-1 also downregulates inducible nitric oxide synthase,[24] and nitric oxide has been shown to inhibit neointima formation.[25] Thus the antagonism of ET-1 might augment nitric oxide formation, which might contribute to the observed effects of BSF 302146.

The early administration of the ET_A antagonist might exert a further beneficial effect on vein graft patency in the longer term through the inhibition of atherogenesis, which, when superimposed on neointima formation, leads ultimately to vein graft failure.[2,3] Increased ET-1 content and ET_A receptor density has long been associated with atherogenesis.[12,13] In an animal model the ET_A receptor antagonist BMS 182874 has been shown to reduce early atherosclerosis.[26] Recent studies have demonstrated that nonspecific ET-1 receptor antagonism prevents early proatherogenic events in cultured human VSMCs[27] and that ET-1 promotes the adhesion of monocytes to the endothelium.[28]

In conclusion, the potent inhibitory effect of BSF 302146 on porcine vein graft thickening helps confirm that that ET-1 and ET_A receptor subtypes are involved in medial thickening and neointima formation in saphenous vein grafts. It is also concluded that the effects of BSF 302146 on vein graft morphology are mediated principally through the inhibition of VSMC proliferation and migration. The administration of this ET_A antagonist might therefore prove effective in preventing late vein graft failure in humans and clinical trials would seem to be warranted.

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